Increased Expression of miR-487b Is Associated With Poor Prognosis and Tumor Progression of HBV-Related Hepatocellular Carcinoma

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Background. Increasing evidence has demonstrated the involvement of microRNAs in the pathogenesis of hepatitis B virus (HBV)–related hepatocellular carcinoma (HCC). The aims of this study were to analyze whether miR-487b can be used as a diagnostic and prognostic biomarker for HBV-related HCC and to explore its effect on the biological function of HCC.

Methods. The expression levels of miR-487b in the serum of all subjects were measured by real-time quantitative fluorescence polymerase chain reaction. The diagnostic value of miR-487b in serum was assessed using the receiver operating characteristic (ROC) curve. The relationship between miR-487b and the clinical data of patients was analyzed using the chi-square test. The prognostic value of miR-487b in HCC was assessed by Cox regression analysis and Kaplan-Meier survival. Moreover, CCK-8 and Transwell assays were performed to investigate the effect of miR-487b on HBV-related HCC function.

Results. Our data indicated that miR-487b in HCC patients was significantly higher than in chronic hepatitis B (CHB) patients and healthy controls. Meanwhile, the ROC curve showed that miR-487b had high specificity and sensitivity in the diagnosis of HBV-related HCC. MiR-487b can significantly distinguish between HCC patients and healthy controls and can differentiate HCC patients from CHB patients. Cox regression analysis showed that miR-487b was an independent risk factor. Overexpression of miR-487b was associated with Tumor Node Metastasis stage stage and Barcelona Clinic Liver Cancer stage in HCC patients. Cell function experiments demonstrated that upregulated miR-487b promoted cell proliferation, migration, and invasion.

Conclusions. Combined the results of the current study demonstrate that the upregulation of serum miR-487b may serve as a promising noninvasive diagnostic biomarker for HBV-related HCC.

Keywords. diagnosis; hepatitis B virus; hepatocellular carcinoma; miR-487b; prognosis.

Hepatocellular carcinoma (HCC) is a type of malignant cancer with features like a rapidly progressing, highly malignant tumor with poor prognosis and high mortality [1–3]. Various factors are closely related to the onset and development of HCC. Hepatitis B virus (HBV) infection is one of the main risk factors for the development of liver cancer, whose high infection rate leads to a high prevalence of HCC [4]. Currently, numerous serum markers have been used for HCC detection in clinical terms, such as alpha-fetoprotein (AFP) [5]. The sensitivity and specificity of AFP for the detection of HBV-related HCC at an early stage are unsatisfactory [6, 7]. Meanwhile, limited treatment options for late-stage HBV lead

to the high prevalence of HCC [4]. Therefore, to increase the overall survival of HBV-related HCC patients, it is urgent that noninvasive and effective biomarkers for early detection be discovered.

MicroRNAs (miRNA), a class of noncoding RNAs that contain ~19–25 nucleotides, are known to function in the regulation of gene expression [8]. Multiple studies have also confirmed that miRNAs are involved in a variety of cellular processes such as proliferation, differentiation, and apoptosis [9]. Abnormal expression of miRNAs often occurs in tumor tissues, showing their critical role in tumorigenesis [10–14]. miRNAs have been identified both in tissues and body liquids like blood. Some researchers have suggested that cancer tissues are an important source of circulating miRNAs [15]. Moreover, miRNAs have shown high stability in the blood even after repeated freezing-thawing operations, which makes circulating miRNAs a valuable noninvasive biomarker for cancer detection [16]. Some miRNAs have been identified to dysregulate expression and act as either oncogenes or tumor suppressors in HBV-related HCC [16–18]. Wang et al. studied the microRNA profile in HBV-induced infection and HCC and found that miRNAs could be involved in the development of HBV-related HCC. The expression level of miR-487b in HBV-related tumors was significantly

higher than in the nontumor group [19]. However, the clinical role of miR-487b in HBV-related HCC and its regulatory mechanisms remain largely unknown.

Therefore, our study explored the expression of miR-487b in HBV-related HCC patients and investigated its potential clinical diagnostic value. Then we analyzed the role of miR-487b in the biological behavior of HBV-related HCC cells. The obtained data confirm that miR-487b is a potential diagnostic biomarker in HBV-related HCC.

METHODS

Research Objects

The samples of 87 HBV-related HCC patients, 68 patients with chronic hepatitis B (CHB), and 70 healthy controls were obtained from Jinan Infectious Disease Hospital from June 2008 to June 2014. The healthy control group had no history of liver disease or viral hepatitis. Blood samples were collected from patients with CHB when the disease was active. Chronic HBV infection was determined using the hepatitis B surface antigen enzyme-linked immunosorbent assay kit (InTec, Xiamen, China). None of the patients had undergone any surgery, radiofrequency ablation, chemoradiotherapy, or immunotherapy before the collection of samples. A histopathology examination was used to assess the diagnosis of HCC. The blood samples were obtained from all patients at initial diagnosis; all patients did not receive antiviral therapy. Additionally, we estimated HCC patients according to Barcelona Clinic Liver Cancer (BCLC) stage [20, 21]. The data of basic clinical characteristics were all recorded. After a few minutes of rest, the collected blood samples were centrifuged. The upper serum was collected and kept at –80°C. Moreover, the HBV-related HCC patients in our study received a radical hepatectomy following the Chinese guidelines for the diagnosis of primary liver cancer and the clinical experience of surgeons. Follow-up was performed through regular outpatient visits or telephone interviews. If someone could not be contacted after 3 consecutive attempts, they were considered lost to follow-up. The follow-up duration was from the date of operation to the date of death or the end of the last follow-up. The last follow-up was in June 2019. As shown in Table 1, the clinicopathological features of the patients were recorded.

All the methods were performed according to the relevant guidelines, including any relevant details. Written informed consent was obtained from each patient. The study was approved and authorized by the ethics committee of Jinan Infectious Disease Hospital, and all procedures were performed in accordance with the Helsinki Declaration.

Cell Culture and Transfection

The Hep3B cell line was procured from the Chinese Academy of Science Cell Bank (Shanghai, China). This cell line was derived

Table 1. The Demographic and Clinical Characteristics of the Subjects

Characteristic	HCC (n = 87)	CHB (n = 68)	Healthy Control (n = 70)
Gender			
Male	61	49	38
Female	26	19	22
Age, y			
Mean ± SD	59.95 ± 8.14	57.66 ± 7.08	58.82 ± 6.69
AFP value			
Mean ± SD	270.76 ± 274.79	23.47 ± 5.84	8.41 ± 1.90
HBsAg status			
HBsAg⁺	87	68	0
HBsAg⁻	0	0	70
ALT, IU/L			
Mean ± SD	64.51 ± 18.46	45.92 ± 6.89	31.71 ± 4.47
AST, IU/L			
Mean ± SD	66.71 ± 18.98	49.51 ± 7.34	33.13 ± 4.76
Liver cirrhosis			
Present	46	37	
Absent	41	31	
Tumor diameter, cm			
<5	56		
≥5	31		
Differentiation			
Poor	40		
Moderate + well	47		
TNM stages			
I + II	50		
III	37		
BCLC stage			
A	52		
B	35		

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; TNM, Tumor Node Metastasis.

from a chronic HBV carrier and was suitable for transfection. The cells were cultured as recommended with high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and penicillin-streptomycin (100 IU/mL, Invitrogen, Shanghai, China). The cells were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. miR-487b mimic, miR-487b inhibitor, and negative control miRNA (mimic-NC and inhibitor NC) were obtained from RiboBio (Guangzhou, China) for the in vitro regulation of miR-487b. The Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA.) was used for all of the transfections based on the manufacturer's instructions. A follow-up experiment was performed after 24 hours.

RNA Extraction and Real-time Fluorescence Quantitative Polymerase Chain Reaction

According to the manufacturer's instructions, total RNA was isolated from the serum sample cell lines using the TRIzol Reagent and mRNA Pure Mini Kit (CWBiotech, Beijing, China). Then

the separated RNA was reverse-transcribed into cDNA using the miRNA cDNA Synthesis Kit (CWBiotech, Beijing, China). After that, real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) was performed by the miRNA qPCR Assay Kit (CWBiotech, Beijing, China) using the 7300 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The relative levels of miR-487b were normalized to the levels of cel-miR-39–3p (internal standard), and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of miRNA.

Cell Proliferation Assay

The CCK-8 assay was performed to explore the effect of miR-487b on the proliferation of HBV-related HCC cells. Briefly, treated and untreated cells were seeded in 96-well plates with a certain concentration. At a preset time point (incubation time of 0, 24, 48, 72 hours), 10 µL of CCK-8 reagent was added to each well and the mixture was incubated for 1 hour. After treatment, optical density values (ODs) at a wavelength of 490 nm were measured by a microplate reader (Bio-Rad, Hercules, CA, USA), which expressed the proliferation capacity of the cells.

Cell Migration and Invasion Assay

The migration and invasion ability of cells was estimated by Transwell assay using 8-µm pore Transwell chambers in 24-well plates. For the migration assays, the transfected cells in the logarithmic growth stage were seeded in the upper chamber at a density of 5×10^4/mL; 500 µL of DMEM with 10% FBS was added to the lower chambers. The unmigrated cells on the upper side were removed, while the migrated cells were fixed with methanol and stained with 0.5% crystal violet, followed by culturing for 24 hours. Five random fields were selected under the microscope to calculate the number of cell migrations. Moreover, the method of invasion was similar to migration, as the upper chamber of the Transwell chamber had previously been coated with 50 µL of Matrigel before the addition of the cells, and cells were inoculated.

Statistical Analysis

The experimental data were expressed as mean ± SD. Statistical analysis of all data was conducted using SPSS 24.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.04 (GraphPad, San Diego, CA, USA). Statistical differences between groups were compared using the Student t test and 1-way analysis of variance. The relationship between miRNA expression and clinical parameters of patients was analyzed using the χ^2 test. A receiver operating characteristic (ROC) curve was performed, and the area under curve (AUC) with 95% CI was calculated to assess the diagnostic value of serum miR-487b expression in patients. The prognostic significance of miR-487b was confirmed by Kaplan-Meier analysis and Cox regression analysis. Each experiment was run at least in triplicate, and $P < .05$ was considered statistically significant.

RESULTS

Demographic and Clinical Characterization of the Study Population

Table 1 shows the demographic and clinical characteristics of the subjects, including 87 HBV-related HCC patients, 68 CHB patients, and 70 healthy controls. Moreover, there was no statistical difference in age or gender among the 3 groups.

Increased Serum Levels of miR-487b in HCC Patients

Firstly, we detected the expression level of miR-487b in the serum of healthy subjects, CHB patients, and HBV-related HCC patients using qRT-PCR. The result indicated that the expression level of miR-487b in the serum of CHB patients significantly increased compared with the healthy control group ($P < .001$) (Figure 1). Moreover, the expression level of serum miR-487b in HCC patients was significantly higher than that in healthy controls and CHB patients ($P < .001$).

Diagnostic Potential of Serum miR-487b Levels in HCC

To explore whether miR-487b in serum could be used as a diagnostic marker for HBV-related HCC, a ROC curve was performed. As shown in Figure 2, miR-487b was able to discriminate the HBV-related HCC patients from the healthy patients. The area under the curve (AUC) for miR-487b was 0.929; miR-487b had a sensitivity of 83.9% and a specificity of 92.8% at a cutoff value of 1.646.

To further determine the potential diagnostic value of miR-487b in distinguishing HBV-related HCC patients from CHB patients, we analyzed the ROC curve. The AUC for miR-487b was 0.856, the cutoff value was 1.836, and the sensitivity and specificity were 75.9% and 89.7%, respectively. These results show that miR-487b was a useful biomarker to distinguish HCC patients from CHB patients.
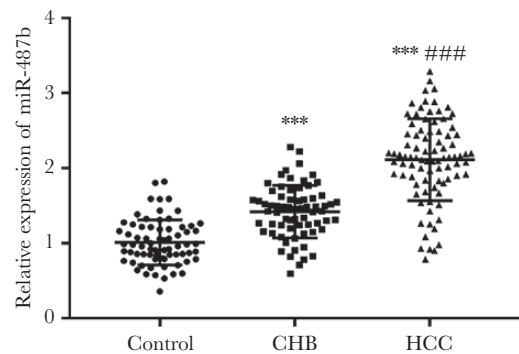
Figure 1. The expression levels of serum miR-487b in different patients were detected by qRT-PCR. Serum miR-487b levels were significantly higher in patients with HBV-related HCC than in healthy controls or CHB patients. $^{***}P < .001$, compared with healthy controls; $^{###}P < .05$, compared with CHB group. Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; qRT-PCR, quantitative reverse transcription polymerase chain reaction.
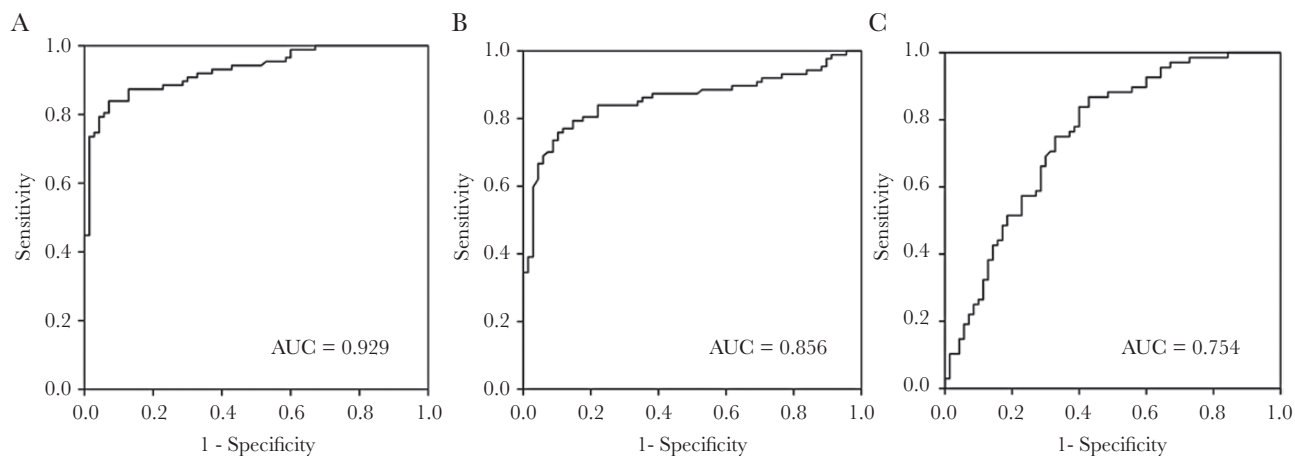
Figure 2. An ROC curve was used to identify the diagnostic value of serum miR-487b in patients. A, HBV-related HCC patients and healthy controls. B, HBV-related HCC patients and CHB patients. C, CHB patients and healthy controls. Abbreviations: AUC, area under the curve; CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ROC, receiver operating characteristics.

Ultimately, we examined whether serum miR-487b could differentiate healthy controls from CHB patients. The AUC of serum miR-487b was only 0.754. And miR-487b had a sensitivity of 86.8% and specificity of 57.1% at the cutoff value of 1.060.

miR-487b Was Correlated With the Clinicopathological Features of HBV-Related HCC Patients

The HBV-related HCC patients were divided into the relatively low-expression group and the relatively high-expression group based on the median value of miR-487b expression in serum. Then we analyzed the relationship between miR-487b expression and the clinical parameters of the patients. Table 2 summarizes the results of the chi-square test: The expression level of miR-487b was closely correlated with TNM ($P = .005$) and BCLC stage ($P = .004$), and in patients with high miR-487b expression, a larger proportion of cases were in BCLC stage B and TNM stage III. However, there was no significant difference in correlation with age, gender, serum AFP level, tumor diameter, liver cirrhosis, alanine aminotransferase (ALT) level, aspartate aminotransferase (AST) level, and differentiation ($P > .05$).

miR-487b Was Correlated With Poor Prognosis in HBV-Related HCC Patients

To explore the relationship between miR-487b expression and prognosis, Kaplan-Meier analysis and Cox regression were performed. As shown in Figure 3, the survival rate of patients with low expression of miR-487b in serum was significantly higher than that of patients with high expression of miR-487b (log-rank test $P = .018$). Moreover, Cox regression results showed that miR-487b (hazard ratio [HR], 2.846; 95% CI, 1.139–7.114; $P = .025$) and TNM stage (HR, 2.703; 95% CI, 1.181–6.187; $P = .019$) were independent prognostic factors for HBV-related HCC (Table 3).

miR-487b Regulated HBV-Related HCC Cells' Proliferation, Migration, and Invasion In Vitro

Mimics and inhibitors of miR-487b were transfected in Hep3B to determine the function of miR-487b in HBV-related HCC. To verify the transfection efficiency, the expression level of miR-487b in transfected cells was detected by qRT-PCR assay. The results demonstrated that the transfection efficiency of miR-487b in Hep3B was highest ($P < .001$) (Figure 4A). The CCK-8 assay was used to detect the effect of miR-487b on the proliferation of Hep3B. Further analysis demonstrated that miR-487b mimic can significantly increase the proliferation of Hep3B, while miR-487b inhibitor can significantly inhibit the proliferation of cells ($P < .001$) (Figure 4B). To further detect the migration and invasion of HCC cells, Transwell assays were performed. The result showed that the downregulation of miR-487b significantly inhibited the migration and invasion ability of Hep3B cells, while the overexpression of miR-487b promoted the migration and invasion ability of cells ($P < .001$) (Figure 4C, D).

DISCUSSION

The HBV is one of the most common pathogens and is the most significant pathogenic factor of HCC [17]. The occurrence of HBV-related HCC is a complex process involving multiple factors. Despite continuous progress in prevention, screening, diagnosis, and treatment, HCC morbidity and mortality rates show a sustained upward trend. The products and mutations of HBV may disrupt normal cell signal transduction pathways. Several miRNAs have been identified to be differentially expressed in HBV-related HCC patients, some of which have emerged as novel noninvasive biomarkers for the detection and prognosis of HCC [22]. As the study by Gao et al. reported, the expression of miR-429 was downregulated in HBV-HCC tissues and cells compared with controls [17]. Qiao et al. showed that

Table 2. Correlation Between miR-487b Expression Levels and Clinical Features

| Parameters	Cases No. (n = 87)	miR-487b Expression Low (n = 41)	High (n = 46)	P Value
Gender				
Male	61	29	32	.906
Female	26	12	14	
Age, y				
<60	29	11	18	.224
≥60	58	30	28	
Tumor size, cm				
<5	56	29	27	.242
≥5	31	12	19	
AFP, ng/mL				
<20	12	6	6	.830
≥20	75	35	40	
Liver cirrhosis				
Present	46	18	28	.114
Absent	41	23	18	
ALT				
Normal	13	9	4	.083
Elevated level	74	32	42	
AST				
Normal	11	8	3	.069
Elevated level	76	33	43	
Differentiation				
Poor	40	22	18	.175
Well + moderate	47	19	28	
BCLC stage				
A	52	31	21	.004
B	35	10	25	
TNM stage				
I & II	50	30	20	.005
III	37	11	26	

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; TNM, Tumor Node Metastasis.

miR-122 and miR-22 were downregulated in HBV-related HCC patients [23]. Another study demonstrated that serum miR-18a was significantly higher in HBV patients with HCC than healthy controls and confirmed that miR-18a might serve as a novel and potential noninvasive biomarker for HBV-related HCC screening [18]. In this study, serum levels of miR-487b were shown to be highly expressed in CHB patients and HBV-related HCC patients compared with the healthy control group, which is consistent with the results of previous study [19]. It is known that cellular miRNAs can be released into the circulation, and circulating miRNA levels are also affected in HCC. The present results indicate a high level of miR-487b in the serum of HBV-related HCC patients. These findings demonstrate that miR-487b might function as an autocrine/paracrine factor in HBV-related HCC and enhance the survival of tumor cells. Interestingly, it has been suggested that antiviral therapy may influence the expression of miRNAs. Therefore, it would be worthwhile to investigate miR-487b expression changes after antiviral therapy in future studies. Additionally, information about

the length of HBV infection was not collected in the present study, which may influence the level of miR-487b. Further studies are needed to examine this in the future.

Previous research has shown that abnormal miRNA expression in serum could be used as a biomarker for cancer. Work by Chen et al. established that miR-487b was significantly downregulated in metastatic tissues compared with matched tumor tissues and that miR-487b can be regarded as a biomarker for early colorectal cancer diagnosis and a criterion to judge metastasis [24]. Another study by Wang et al. illustrated that miR-487b played an oncogenic role in osteosarcoma progression via directly targeting TRAK2, which could advance the development of cancer treatment [25]. In the present study, considering the dysregulation of miR-487b in the serum of CHB and HBV-related HCC patients, ROC curves were constructed to evaluate the diagnostic value of miR-487b. It was found that serum miR-487b might be a promising diagnostic biomarker for the early diagnosis of CHB. Furthermore, serum miR-487b was able to distinguish HCC patients from CHB
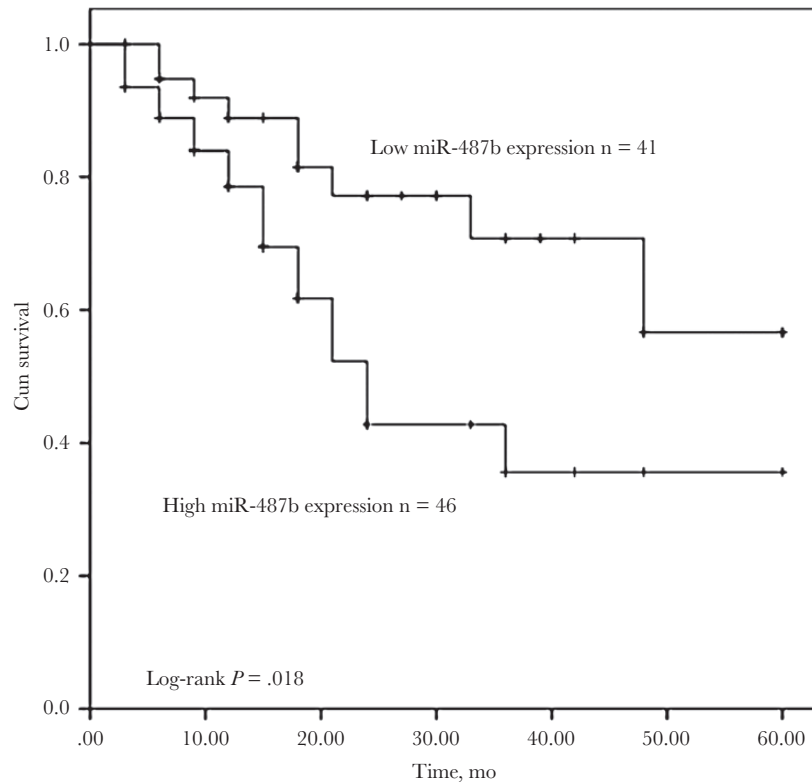
Figure 3. Kaplan-Meier survival curves of overall survival in HBV-related HCC patients based on expression of miR-487b. HCC patients with higher expression of miR-487b had poorer overall survival than those with low expression (log-rank *P* = .018). Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

patients. These results suggest that miR-487b could be used as a promising noninvasive diagnostic biomarker, which could significantly distinguish HBV-related HCC patients from healthy control populations or CHB patients. To our knowledge, this is the first study to preliminarily propose the diagnostic potential of serum miR-487b in screening for HBV-related HCC. Furthermore, Kaplan-Meier analysis and Cox regression were performed to assess the role of miR-487b in the prognosis of HBV-related HCC patients. It was observed that patients with a high expression of miR-487b had shorter survival rates than those with low miR-487b expression. These findings demonstrate the crucial role of miR-487b in the occurrence and development of HBV-related HCC.

Then, we further explored the role of miR-487b in HBV-related HCC cell behaviors in vitro. Notably, previous studies have reported the regulatory role of miR-487b in various types of cancer cells, including cell proliferation, migration, and invasion. For instance, Feng et al. reported that miR-487b promoted human umbilical vein endothelial cell proliferation, migration, invasion, and tube formation through regulating THBS1, which could act as a potential therapeutic option for neurovascular disease [26]. In the current study, the Hep3B cell line was used for in vitro experiments, and the level of miR-487b was regulated by cell transfection. As expected, the downregulation of miR-487b significantly inhibited cell proliferation, migration,

and invasion, which supported the results concluded in clinical samples. These results suggest that miR-487b might be a potential oncogene in the progression of HCC.

There are some shortcomings in the study. First, the relatively small sample size is a major limitation of this study. As this was a retrospective study, potential selection bias cannot be ruled out. A further study with a larger cohort of patients is

Table 3. Multivariate Cox Analysis of miR-487b in HBV-Related Hepatocellular Carcinoma Patients

Parameters	Multivariate Analysis		
	HR	95% CI	*P*
miR-487b	2.846	1.139–7.114	.025
Gender	1.732	0.642–4.667	.278
Age	0.501	0.179–1.403	.188
AFP	1.067	0.367–3.101	.905
Liver cirrhosis	1.567	0.674–3.640	.296
ALT	2.947	0.369–8.510	.308
AST	2.413	0.793–7.348	.121
Tumor size	1.155	0.500–2.667	.736
Differentiation	2.145	0.824–5.584	.118
BCLC stage	1.790	0.744–4.309	.194
TNM stage	2.703	1.181–6.187	.019

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HR, hazard ratio; TNM, Tumor Node Metastasis.
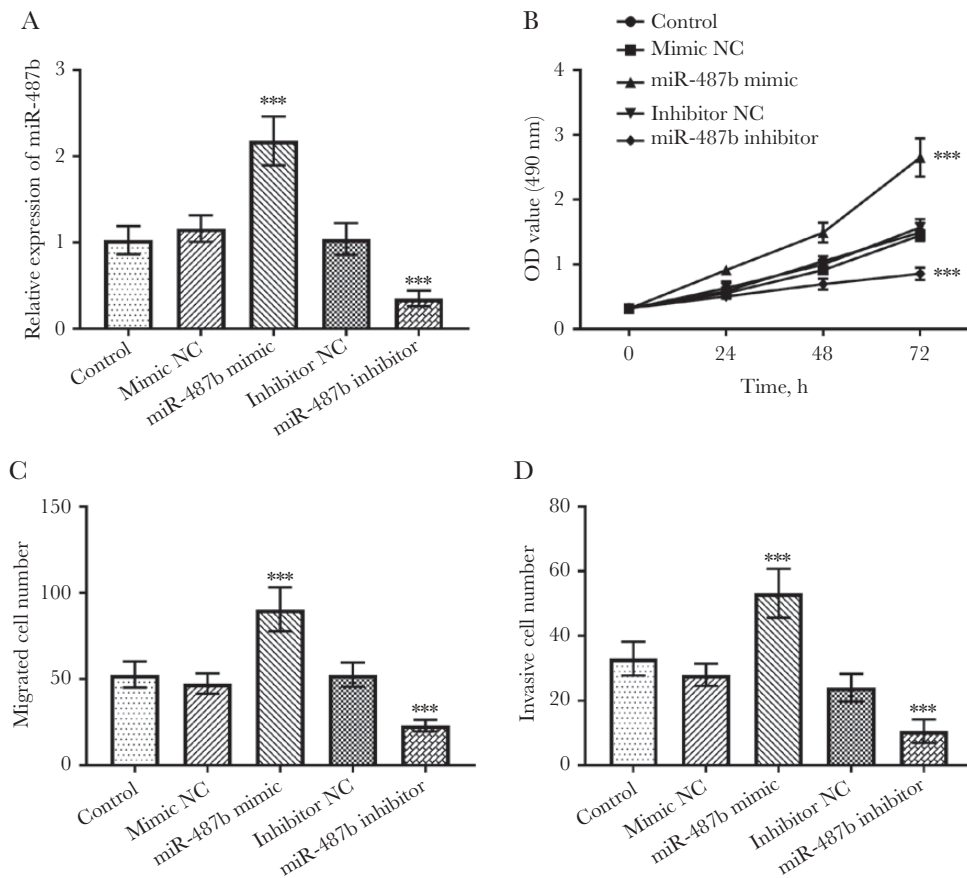
Figure 4. Effects of miR-487b on proliferation, migration, and invasion of HCC cells. A, Changes in miR-487b levels in cells after transfection with miR-487b mimic and inhibitors were detected by qRT-PCR. B, Cell proliferation was detected by CCK-8 assay. miR-487b mimic significantly promoted cell proliferation, while miR-487b inhibitors significantly inhibited cell proliferation. C and D, The Transwell assays for cell migration and invasion. Overexpression of miR-487b significantly promoted cell migration and invasion, while inhibition of miR-487b significantly inhibited cell migration and invasion. ***$P < .001$, compared with control group. Abbreviations: HCC, hepatocellular carcinoma; NC, negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

thus needed to confirm the present findings. In addition, the stability of miR-487b may be affected by freezing, thawing, or storage, and bias may be introduced through the use of frozen blood samples. Besides, as the high AUC value reflects the diagnostic value of miR-487b, a blinded validation set is needed to calculate the diagnostic power of miR-487b in HCC and HBC in future studies.

In conclusion, a series of experiments suggested that miR-487b might be a promising potential biological diagnostic and prognostic biomarker for HBV-related HCC and could affect tumor progression by inhibiting cell proliferation, migration, and invasion. The present data provide a preliminary elucidation about the role of miR-487b in HBV-related HCC. Further study is needed to elucidate the mechanisms of miR-487b in HBV-related HCC, and in vivo experiments should be taken into account.

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